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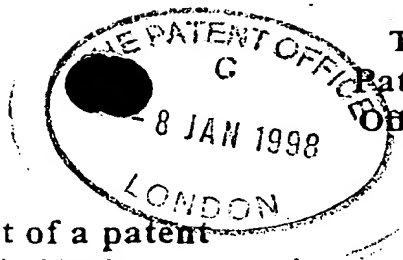

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2. Patent
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3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

The University of Liverpool,
Senate House,
Abercromby Square,
Liverpool,
L69 3BX

Patents ADP number (*if you know it*)

England. 7983550 1

If the applicant is a corporate body, give the country/state of its incorporation

ENGLAND.

4. Title of the invention — PROTEOME ANALYSIS

5. Name of your agent (*if you have one*) — W.P.THOMPSON & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Coopers Building,
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L1 3AB.

Patents ADP number *(if you know it)* — 0000158001

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Country	Priority application number *(if you know it)*	Date of filing *(Day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(Day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'yes' if:*

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a) any applicant named in part 3is not an inventor, or
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Description	12
Claims(s)	
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11. I/We request the grant of a patent on the basis of this application

Signature Date07/01/98

W.P.THOMPSON & CO.

12. Name and daytime telephone number of person to contact in the United Kingdom

D.C.SCHILLER.
0151-709-3961

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DESCRIPTION

PROTEOME ANALYSIS

The present invention relates to proteome analysis, and methods for use in measuring protein expression.

Eukaryotic cells typically express several thousand different proteins, and each of these may be subject to a wide variety of dynamic post-translational modifications, effectively increasing the number of different proteins present. Although not yet being undertaken on the same scale as the DNA sequencing programmes, a number of projects aimed at characterising all the PROTEins expressed by the genOME of an organism - the 'proteome' - are now under way.

In these projects, complex mixtures of proteins are resolved by two-dimensional (2-D) gel electrophoresis, and individual proteins characterised by a growing array of methods.

In many instances, the methods used to characterise proteins present on a 2-D gel pattern are dependent on the existence of DNA sequence data - genome and proteome analysis are therefore (intimately) linked.

However, DNA sequence data alone cannot be used to build a 'molecular definition' of a cell. The DNA sequence data reveals little or nothing about the level of expression of proteins, the protein isoforms that may be produced from each gene (by alternative splicing) or the extent to which protein(s) are post-translationally modified. Nor

can they provide information on the cellular or subcellular distribution or proteins. It is precisely this type of information that proteome analysis will provide.

There are, at present, two (concurrent) phases to proteome analysis. First, all the proteins expressed by a particular organism, tissue or cell under 'normal' conditions are identified and their position on 2-D gels mapped - this defines the 'constitutive' proteome. The information can be used to generate a 2-D reference map and database for the organism, tissue or cell examined.

In its 'second phase', proteome analysis involves the measurement of changes in the proteome that occur in response to changing physiological or pathological conditions. This includes analysis of the quantitative changes in protein expression, changes in post-translational modifications and, where appropriate, changes in subcellular localization of proteins.

Whilst 2-DE serves as an effective tool for the resolution and identification of proteins it has many limitations and is unlikely to be applicable to this 'second phase' of proteome analysis. Alternative methods for measuring protein expression on a proteome scale will be developed and it is likely that such methods will be dependent on the availability of protein affinity ligands which recognise specifically each of the proteins expressed from a genome.

It is an aim of the present invention to provide a high

throughput method to produce 'catalogued' libraries of protein affinity ligands which will open the way for novel approaches of measuring protein expression on a proteome scale.

The term protein affinity ligand as used herein refers to a ligand which binds to an epitope of a protein or a modified protein and includes binding sites of, for example, phosphorylated, fatty acylated, acetylated, ADP-ribosylated and glycosylated proteins. Such protein affinity ligands might include antibodies, peptide or peptoid moieties, oligonucleotides, modified oligonucleotides or any chemical which binds to a protein with specificity. Chemicals might include those selected from combinatorial libraries. In one embodiment, the invention is directed towards antibodies as the protein affinity ligands, although other affinity ligands may be used.

According to the present invention there is provided a method of screening for one or more protein affinity ligands comprising:

(a) resolving a complex protein mixture;

(b) subjecting the resolved protein(s) to peptide mass fingerprinting to obtain a peptide profile;

(c) utilizing one or more of the resolved proteins or the complex protein mixture to either

(i) generate one or more monoclonal antibodies thereto or;

(ii) select one or more affinity ligands from a library;

(d) adding the complex protein mixture to either

(i) the one or more monoclonal antibodies generated in Step (c) (i), and selecting those protein(s) which bind the one or more monoclonal antibodies or

(ii) the one or more affinity ligands selected in step (c) (ii) and selecting those protein(s) which bind the one or more affinity ligands,

and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide profile; and

(e) comparing the profiles obtained in Steps (b) and (d) to select or identify the protein(s) and hence protein affinity ligands.

Preferably the complex protein mixture is resolved by 2D electrophoresis.

In one embodiment of the present invention there is provided a method of generating monoclonal antibodies to one or more targeted proteins comprising the steps of:

(a) resolving a complex protein mixture;

(b) subjecting the resolved protein(s) to peptide mass finger printing to obtain a peptide profile;

(c) utilising one or more of the resolved proteins to generate one or more monoclonal antibodies thereto;

(d) adding the complex protein mixture to the one or more antibodies generated in Step (c), to select those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to

peptide mass fingerprinting to obtain a peptide profile;

(e) comparing the profiles obtained in steps (b) and (d); and

(f) selecting one or more hybridoma clones of interest.

In a second embodiment of the present invention there is provided a method of generating an antibody library comprising the steps of:

(a) resolving a complex protein mixture;

(b) subjecting the resolved protein(s) to peptide mass finger printing to obtain a peptide profile;

(c) utilising the complex protein mixture to generate one or more monoclonal antibodies thereto;

(d) adding the complex protein mixture to the one or more monoclonal antibodies generated in Step (c) to select to those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide profile;

(e) comparing the profiles obtained in steps(b) and (d); and

(f) identifying the monoclonal antibodies of potential interest for a monoclonal antibody library.

In a third embodiment of the present invention there is provided a process for selecting desired members of an affinity ligand library comprising the steps of:

(a) resolving a complex protein mixture;

(b) subjecting the resolved protein(s) to peptide mass finger printing to

obtain a peptide profile;

(c) utilising one or more of the resolved proteins to select one or more affinity ligands from a library;

(d) adding the complex protein mixture to the one or more affinity ligands generated in step (c) to select those proteins which bind the one or more affinity ligands, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide profile;

(e) comparing the profiles obtained in steps (b) and (d); and

(f) selecting one or more affinity ligands of interest.

Hence, the process represents a novel linkage between the resolution and identification by mass spectrometry of individual proteins from complex mixtures with the screening of affinity ligands by mass spectrometry. The most significant advantage of the process is the provision of a method to screen affinity ligands to proteins without requiring pure protein. To date no other screening process which circumvents the need for pure protein has been described.

The present invention includes provision for undertaking those steps in which proteins are selected by a protein affinity ligand and subsequently analysed by mass spectrometry directly on the mass spectrometry targets or alternatively after some additional processing of the selected proteins prior to mass spectrometry.

A number of aspects of the invention are described in more detail, by way of example only, with reference to the following examples

and flow diagrams.

Comparative Example

Current Approach

In a conventional approach to the generation of monoclonal antibodies, an antigen or mixture of antigens to mice or rats is administered and the immune response to the antigen(s) is monitored by ELISA, dot blotting or, less frequently, Western blotting. Having identified animals which have produced an adequate immune response the spleen cells from the animal are fused with cells of a myeloma cell line to generate hybridomas - immortalised cells which produce antibodies. The hybridoma cells are then cloned by limiting dilution and the clones then screened by a range of different screening approaches to determine which cells are producing the desired antibodies. For effective selection of hybridomas the screening process must be robust, rapid and reliable because it is undertaken in parallel with the initial culture of the hybridoma cells. There are three classes of screening strategy: antibody capture assays, antigen capture assays and functional screens. In general, the vast majority of screens are currently undertaken by antibody capture. In this approach the pure antigen bound to a solid surface is used to capture the antibodies and these are then detected with appropriate labelled anti-immunoglobulin antibodies. This process screens for antibodies to an individual protein antigen and requires significant quantities of purified protein. Antigen capture

screens are rarely used unless the protein antigen is available in large quantities - this is because they require labelling of the antigen (often with radioisotopes i.e. ^{125} I).

Thus, although immunisation may require only small amounts of antigen (often less than 1µg is appropriate) the screening process requires relatively large quantities of pure protein. Thus, in the context of current approaches to proteome analysis where 2-DE is used to resolve thousands of proteins in sub-µg quantities existing methods for antibody production are unsuitable.

The process described below provides a novel approach to the widespread production of antibodies or other protein affinity ligands to proteins present in and resolved from complex mixtures. The novel linkage between resolution of complex protein mixtures and the screening of affinity ligands by mass spectrometry provides a powerful approach to the generation of characterised protein affinity ligands.

Example 1

Process directed towards generation of monoclonal antibodies to 'targetted proteins'.

A complex protein mixture is separated by, for example, two dimensional electrophoresis (2-DE).

The 2-DE resolved proteins of interest are subjected to

peptide mass fingerprinting - this gives a characteristic peptide pattern unique to each of the proteins and it is this which is exploited to select antibodies which are capable of binding the proteins.

Antibodies secreted from individual hybridoma clones are allowed to interact with a protein mixture containing the protein for which antibodies are desired. The appropriate antibodies will bind the protein of interest and the protein bound by antibody may then be eluted and subjected to peptide mass fingerprinting. The specificity and sensitivity of the mass spectrometry will allow the identification of the protein(s) of interest and so reveal which hybridoma cells produce antibodies to the protein(s).

This method is described more fully with reference to Fig. 1, which is a flow diagram of the method.

To generate monoclonal antibodies to protein(s) of interest, mammals, for example, rats or mice are immunised (Step C) with individual proteins recovered from, for example, 2-DE gels. The proteins will have previously been subjected to peptide mass fingerprinting (Step B).

After fusing spleen cells with myeloma cell lines to generate hybridomas clones are produced by limiting dilution of the cells and antibodies produced by individual clones screened by mass spectrometry. Thus, monoclonal antibodies are recovered from the tissue culture media and immobilised. To the immobilised monoclonal

antibodies are added the protein mixture (used to generate the original gel) and the proteins which have bound to individual monoclonal antibodies eluted and subjected to peptide mass fingerprinting (Step D). By comparing the profiles (Step E) obtained with the profile of the protein of interest it will be possible to select (Step F) those monoclonal antibodies which are specific for the proteins and hence isolate the relevant clones.

In yet a further improvement the process is streamlined by application of automated (96) well plate handling technology and automated high-throughout mass spectrometry so facilitating the screening of large numbers of individual cell clones.

Example 2

Process directed towards the generation of an antibody library.

This process is described with reference to Fig. 2, which is a flow diagram of the method.

Mice/rats are immunised (Step C) with a complex mixture of proteins and the immune response screened by ELISA using the protein mixture. To establish the 'distribution' of antibodies generated - i.e. the coverage of the polyclonal library, the antiserum is used for Western blotting against 2-DE resolved proteins (of the original complex mixture (Step A)). For those animals which produce antibodies of the desired coverage the spleen cells are fused with a myeloma cell line to generate hybridomas and the antibodies produced by the total pool of

hybridoma cells re-screened by, for example, Western blotting against the 2-DE resolved proteins. This will confirm that suitable antibody producing hybridomas have been generated. The hybridoma mixture is cloned by limiting dilution and antibodies selected (Step D) and screened by mass spectrometry. As with example 1 the profiles can be compared (Step E) and the clones of interest selected (Step F).

The key advantages of the approaches outlined above are:

1. purified protein is not required for screening;
2. monoclonal cell lines are relatively stable and can be archived;
3. antibodies of high affinity will be produced; and
4. the protein mixture used for screening the antibodies can be presented to the antibodies in a form tailored to the likely end application i.e. the proteins may be presented in native or denatured form.

Example 3

Process for selecting phage displayed antibodies.

This process is described with reference to Fig. 3, which is a flow diagram of the method.

Following separation of complex protein mixtures by, for example, 2-DE (Step A), the proteins are Western blotted (Step C) using an antibody phage library with detection of bound phage. Individual protein spots of interest and associated antibody displaying phage are excised from the Western blot, the phage eluted and used to re-infect

E.coli. Individual colonies of E.coli may be propagated (in 96 well plate format), phage antibody secretion induced with IPTG and individual antibodies (PhAbs) recovered. The PhAbs are then immobilised and the complex protein mixture added. PhAbs which bind the protein(s) of interest will be selected (Step D) and identified by peptide mass fingerprinting of the released protein(s). As with Example 1 the profiles can be compared (Step E).

The key advantages of the approach outlined above are:

1. it avoids the typical 4-5 rounds of phage selection (not yet achieved for 2-DE resolved proteins);
2. purified protein is not required for screening;
3. the method also reveals whether any PhAbs which bound to the protein during Western blotting are specific for other proteins in the protein mixture. Each screens will therefore produce PhAbs to at least the protein of interest and potentially others as well;
4. the phage can be archived to generate a stable source of PhAbs (Step F);
5. modification of the Western blotting selection conditions and the conditions under which complex protein mixture is added to and washed from individual PhAbs can be modified to isolate PhAbs of desired properties (i.e. affinity, avidity); and
6. relevant selected phage can be genetically modified to alter properties of PhAbs.

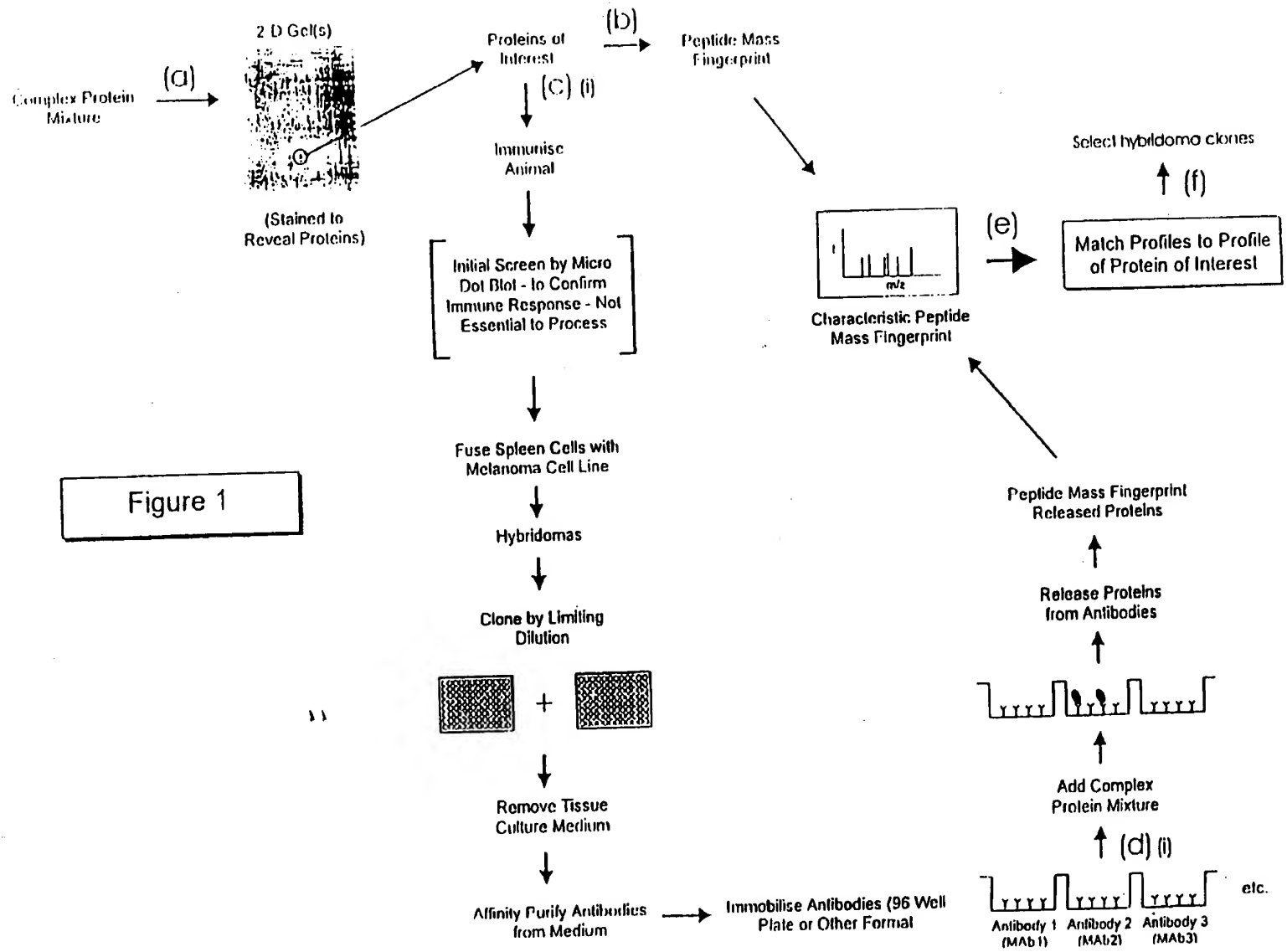

Complex Protein Mixture
(a)
2 D Gel(s)
(Stained to Reveal Proteins)
Proteins of Interest
(b)
Peptide Mass Fingerprint
(c) (i)
Immunise Animal
Initial Screen by Micro Dot Blot - to Confirm Immune Response - Not Essential to Process
Fuse Spleen Cells with Melanoma Cell Line
Hybridomas
Clone by Limiting Dilution
Remove Tissue Culture Medium
Affinity Purify Antibodies from Medium
Immobilise Antibodies (96 Well Plate or Other Format
Antibody 1 (MAb1)
Antibody 2 (MAb2)
Antibody 3 (MAb3)
etc.
(d) (i)
Add Complex Protein Mixture
Release Proteins from Antibodies
Peptide Mass Fingerprint Released Proteins
Characteristic Peptide Mass Fingerprint
(e)
Match Profiles to Profile of Protein of Interest
(f)
Select hybridoma clones

Figure 1

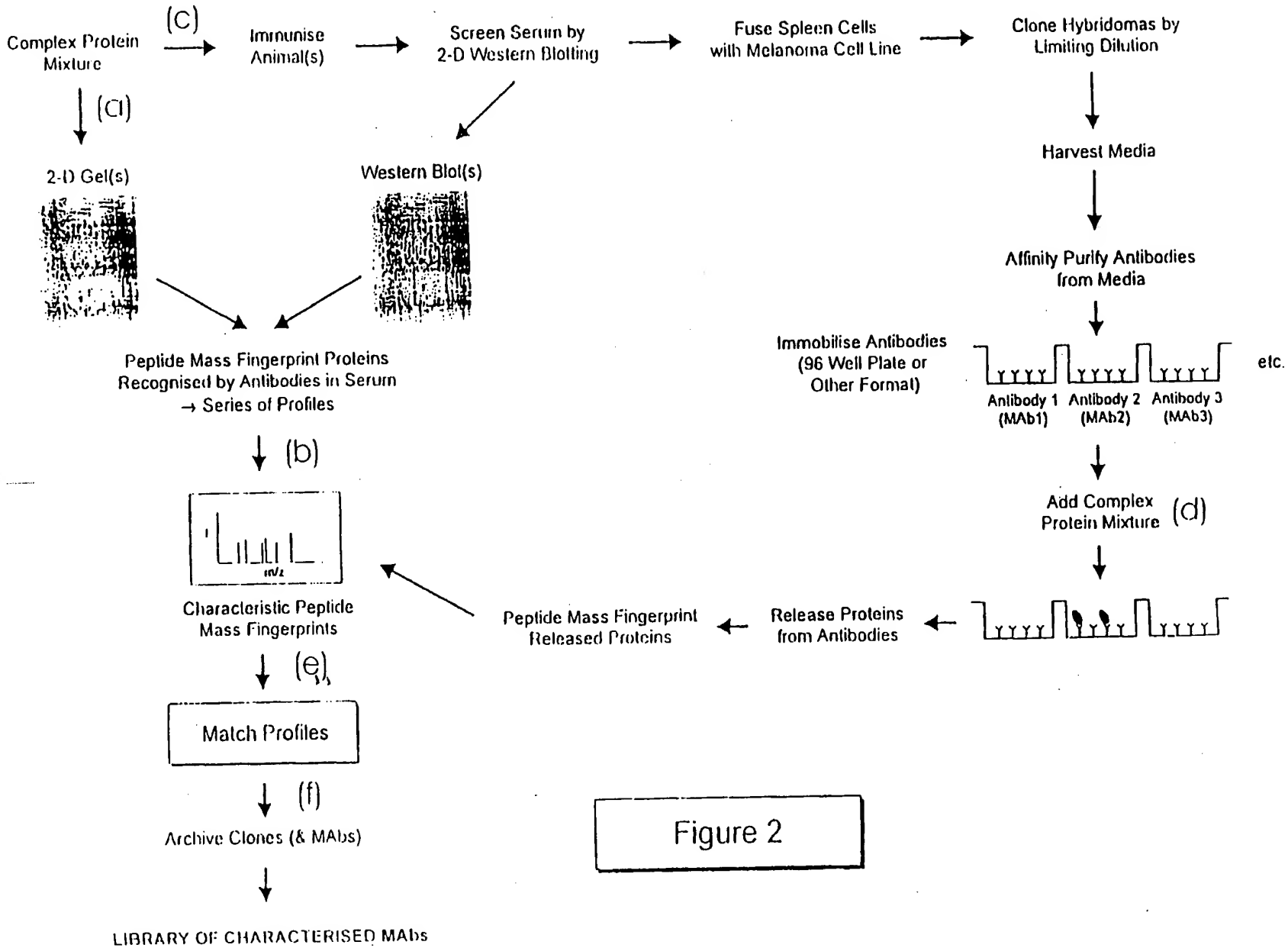
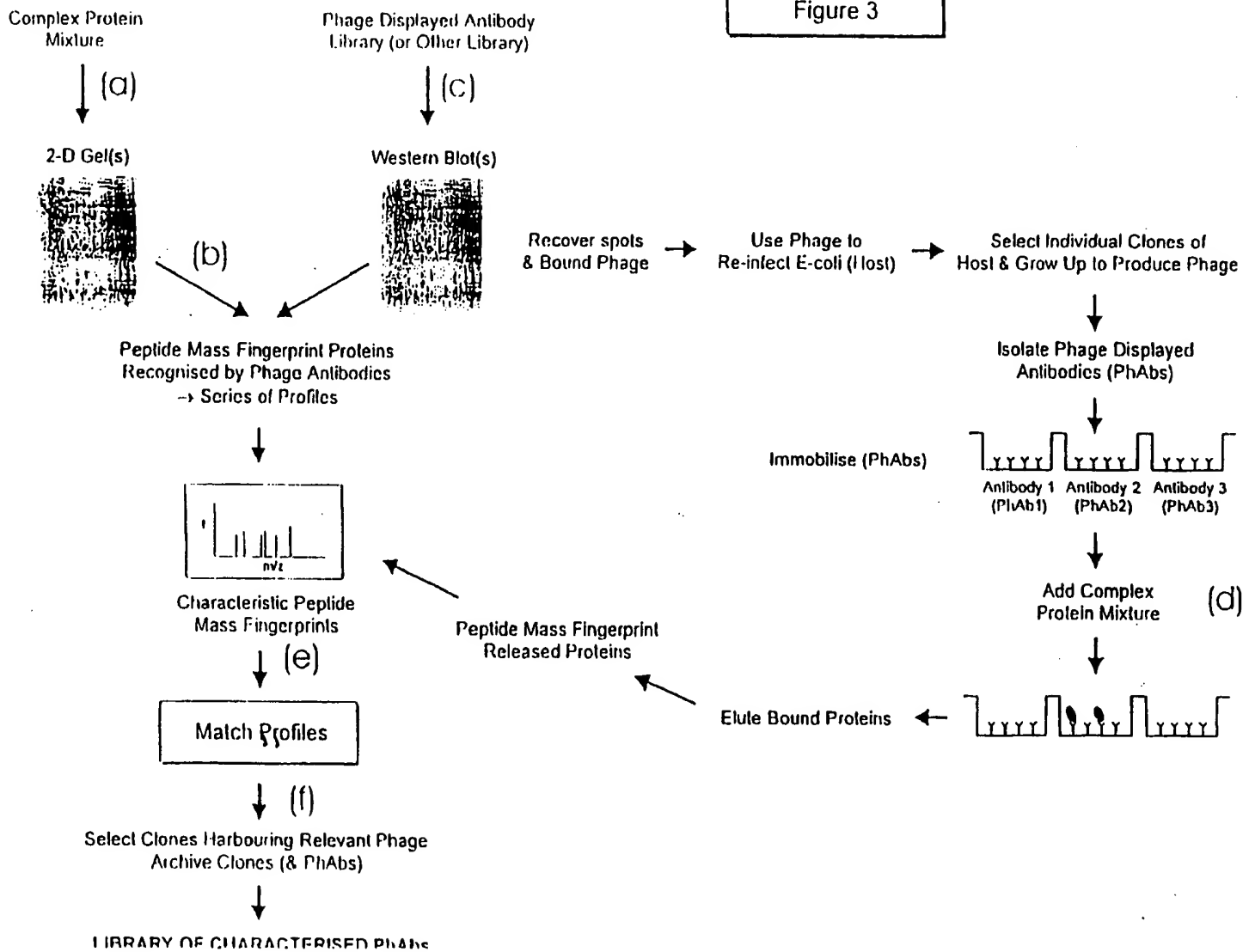

Complex Protein Mixture
(c)
Immunise Animal(s)
Screen Serum by 2-D Western Blotting
Fuse Spleen Cells with Melanoma Cell Line
Clone Hybridomas by Limiting Dilution
(a)
2-D Gel(s)
Western Blot(s)
Harvest Media
Affinity Purify Antibodies from Media
Immobilise Antibodies (96 Well Plate or Other Format)
Antibody 1 (MAb1)
Antibody 2 (MAb2)
Antibody 3 (MAb3)
etc.
Peptide Mass Fingerprint Proteins Recognised by Antibodies in Serum → Series of Profiles
(b)
Add Complex Protein Mixture (d)
Characteristic Peptide Mass Fingerprints
Peptide Mass Fingerprint Released Proteins
Release Proteins from Antibodies
(e)
Match Profiles
(f)
Archive Clones (& MAbs)
LIBRARY OF CHARACTERISED MAbs

Figure 2

Figure 3


Complex Protein Mixture
(a)
2-D Gel(s)
Phage Displayed Antibody Library (or Other Library)
(c)
Western Blot(s)
(b)
Peptide Mass Fingerprint Proteins Recognised by Phage Antibodies → Series of Profiles
Characteristic Peptide Mass Fingerprints
(e)
Match Profiles
(f)
Select Clones Harbouring Relevant Phage Archive Clones (& PhAbs)
LIBRARY OF CHARACTERISED PhAbs
Peptide Mass Fingerprint Released Proteins
Elute Bound Proteins
Add Complex Protein Mixture
(d)
Immobilise (PhAbs)
Antibody 1 (PhAb1)
Antibody 2 (PhAb2)
Antibody 3 (PhAb3)
Isolate Phage Displayed Antibodies (PhAbs)
Select Individual Clones of Host & Grow Up to Produce Phage
Use Phage to Re-infect E-coli (Host)
Recover spots & Bound Phage

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